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Wound Chronicity, Impaired Immunity and Infection in Diabetic Patients

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ABSTRACT

BACKGROUND Diabetic foot ulcers are a common diabetic complication leading to alarming figures of amputation, disability, and early mortality. The diabetic glucooxidative environment impairs the healing response, promoting the onset of a 'wound chronicity phenotype'. In 50% of ulcers, these non-healing wounds act as an open door for developing infections, a process facilitated by diabetic patients' dysimmunity. Infection can elicit biofilm formation that worsens wound prognosis. How this microorganism community is able to take advantage of underlying diabetic conditions and thrive both within the wound and the diabetic host is an expanding research field.

OBJECTIVES 1) Offer an overview of the major cellular and molecular derangements of the diabetic healing process versus physiological cascades in a non-diabetic host. 2) Describe the main immunopathological aspects of diabetics' immune response and explore how these contribute to wound infection susceptibility. 3) Conceptualize infection and biofilm in diabetic foot ulcers and analyze their dynamic interactions with wound bed cells and matrices, and their systemic effects at the organism level. 4) Offer an integrative conceptual framework of wound–dysimmunity–infection–organism damage.

EVIDENCE ACQUISITION We retrieved 683 articles indexed in Medline/PubMed, SciELO, Boline International and Google Scholar. 280 articles were selected for discussion under four major subheadings: 1) normal healing processes, 2) impaired healing processes in the dia-

betic population, 3) diabetic dysimmunity and 4) diabetic foot infection and its interaction with the host.

DEVELOPMENT The diabetic healing response is heterogeneous, torpid and asynchronous, leading to wound chronicity. The accumulation of senescent cells and a protracted inflammatory profile with a pro-catabolic balance hinder the proliferative response and delay re-epithelialization. Diabetes reduces the immune system's abilities to orchestrate an appropriate antimicrobial response and offers ideal conditions for microbiota establishment and biofilm formation. Biofilm–microbial entrenchment hinders antimicrobial therapy effectiveness, amplifies the host's pre-existing immunodepression, arrests the wound's proliferative phase, increases localized catabolism, prolongs pathogenic inflammation and perpetuates wound chronicity. In such circumstances the infected wound may act as a proinflammatory and pro-oxidant organ superimposed onto the host, which eventually intensifies peripheral insulin resistance and disrupts homeostasis.

CONCLUSIONS The number of lower-limb amputations remains high worldwide despite continued research efforts on diabetic foot ulcers. Identifying and manipulating the molecular drivers underlying diabetic wound healing failure, and dysimmunity-driven susceptibility to infection will offer more effective therapeutic tools for the diabetic population.

KEYWORDS Diabetic foot, amputation, infections, biofilms, microbiota

INTRODUCTION

Diabetes mellitus (DM) is characterized by the onset and progression of a constellation of multi-organ complications resulting from multifactorial interactions—including biochemical derangements and epigenetic factors—which ultimately translates to irreversible tissue changes as a response to glucooxidative processes.[1] Of all diabetic complications, the development of diabetic foot ulcers (DFUs) is among the most common and debilitating.[2,3] Classic concepts define DFU as deep tissue damage of the lower limb, frequently preconditioned by, and associated with, neuropathy or peripheral arterial disease.[4] It is recognized as a major and growing public health problem, a scientific challenge and a socioeconomic burden:[5] and remains the main causal factor of lower extremity amputations, disability and early mortality.[6] Armstrong introduced the 'cancer analogy' concept to highlight the fact that five-year mortality rates associated with foot ulceration and amputation surpass those registered for common cancers.[7–10]

IMPORTANCE This article contrasts wound healing processes in healthy individuals and diabetics, establishing and conceptualizing the reciprocal links between diabetic dysimmunity, susceptibility to infection, diabetic foot ulcer chronicity and insulin resistance amplification.

Diabetic glucooxidative stress impairs the healing response and disrupts the flow of overlapping healing phases, ultimately promoting the onset of a 'wound chronicity phenotype'. [11–13] Aside from healing impairment, a common occurrence in diabetic patients is ulcer recurrence after primary closure.[6] These non-healing wounds are a major predisposing factor or entry point for wound infection[11] and accordingly, more than 50% of DFUs become infected.[14] Infection acts as a primary deterrent to physiological healing responses[15] and a risk factor for lower-limb amputation,[16–18] especially when deep tissues and bones are compromised.[19,20] Although diabetics are particularly vulnerable to bacterial infections,[21–23] DFUs have a complex and highly organized polymicrobial community that frequently contributes to undesirable outcomes in DFU-affected individuals. [22] This microbiota–biofilm comprises symbiotic bacteria, yeast and fungal loads and can silently spread, amplify the underlying healing deficit, increase antibiotic resistance, disrupt host metabolism and further dampen immune response.[24–27]

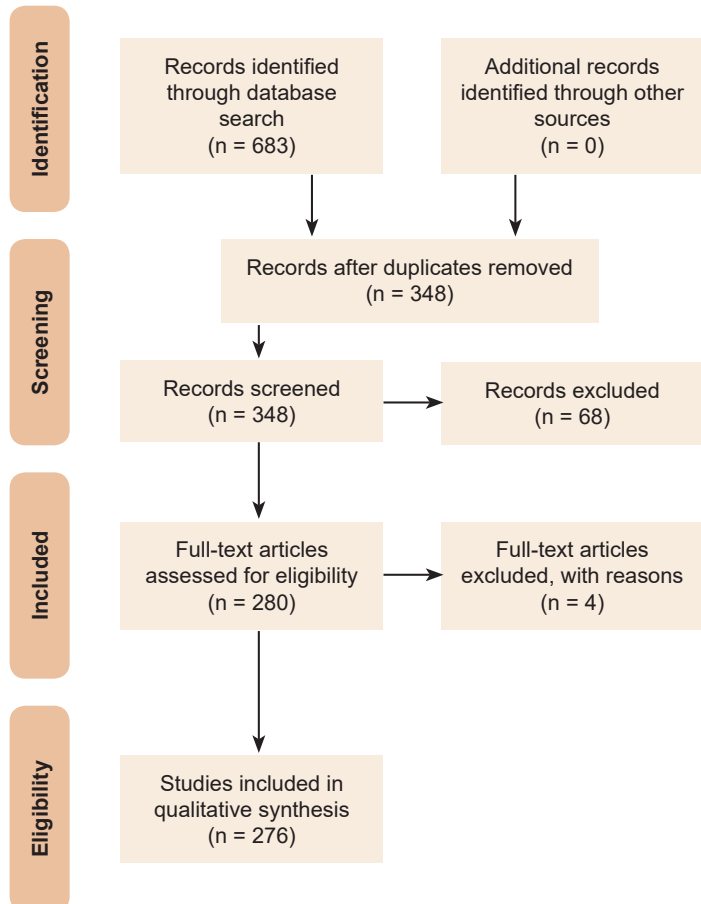
Globally speaking, DM and infection increasingly go together. [28,29] Diabetic individuals are prone to peripheral-tissue infections; given dysregulations in primary surveillance, recognition, activation and neutralization mechanisms within the innate immunity repertoire.[21,30,31] Furthermore, diabetic individuals exhibit antigen presentation failure, contraction of T-cell-mediated immune function[32] and a particular predisposition to bacterial adhesion to epithelial linings.[33,34]

DFU is a unique battlefield where host–microorganism interactions shape ulcer progression. Consequently, numerous studies have addressed the role of biofilm on DFU and its impact within the ulcer bed and the host itself.[35–38] We reviewed this critical issue, given its etiopathogenic relevance to basic aspects of DFU pathology: 1) Why are diabetic persons more susceptible to wound infections? 2) How is DFU biofilm organized? 3) How does a microorganism’s pathogenic potential and concentration impact DFU outcomes? and 4) How does biofilm impair the healing response?

EVIDENCE ACQUISITION

We retrieved articles indexed in Medline/PubMed, SciELO, Boline International, and Google Scholar using the following keywords/phrases: DFU, limb AND amputation, DFU AND infection, DFU AND biofilm, immune system AND diabetic patient, microorganism AND immune system. A total of 683 articles were retrieved and exported a reference manager. Duplicate articles were removed (Figure 1). Our final selection included 280 research and review articles. Titles, objectives and abstracts were carefully screened and reviewed. The search was limited to the English language without date restrictions. All compiled information was structured under four principal headings: 1) a general overview of the normal healing response in a healthy organism; 2) an overview of the cellular and molecular foundations of the impaired healing process in the diabetic population; 3) diabetic dysimmunity; and 4) conceptual definition and pathogenic implications of diabetic foot infection (DFI) in its interaction with the host.

Figure 1. Literature review process



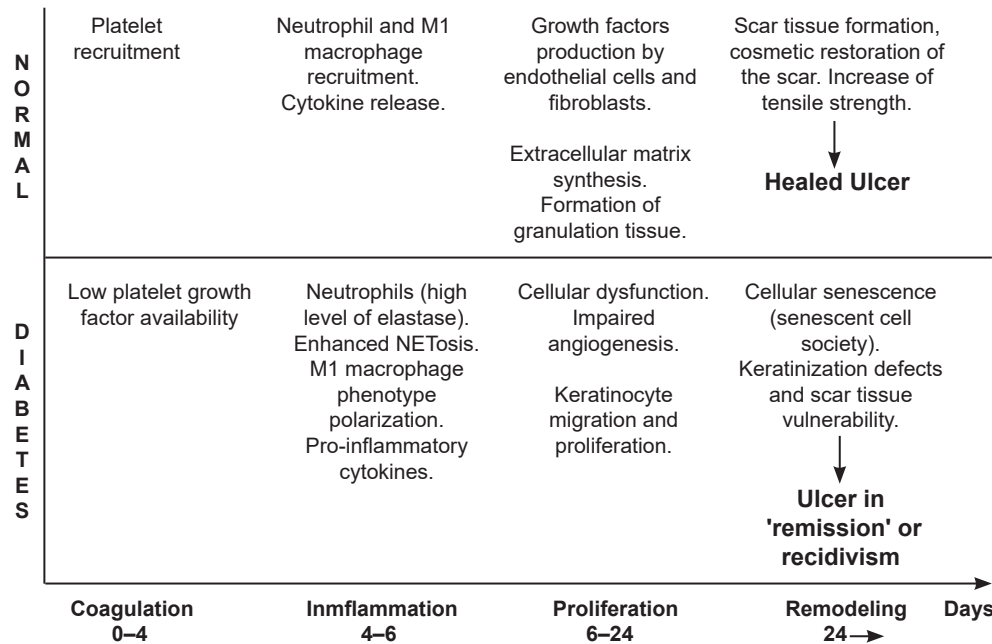
DEVELOPMENT

Brief overview of normal healing response Wound healing is a dynamic and complex process that ultimately results in restoration of anatomic integrity with analogous function.[39–41] Of note, however, skin wound healing represents an evolutionary advantage for organism survival, given its role in restoring barrier function, as well as preventing internal tissue damage and infection dissemination.[42] This evolutionary advantage involves a complex and intricate, but finely regulated, crosstalk between cells and soluble mediators.[43] A normal healing process is made up by four overlapping phases: 1) coagulative, 2) inflammatory, 3) proliferative, and 4) remodeling (Figure 2). Each phase takes place during a temporary window involving a certain cell population, a specific set of cytokines and a particular chemical composition within the extracellular matrix (ECM).[44,45] The coagulation process, aside from ensuring hemostasis, has two other relevant functions: 1) the fibrin clot and fibrinogen byproducts act as a scaffold and chemoattractant for the recruitment and anchorage of inflammatory cells, fibroblasts and other mesenchymal-derived cells that will participate in tissue granulation formation; and 2) platelet degranulation promotes primary growth factors. Platelets represent the first group of resident cells with fibroangiogenic soluble messengers, including platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- β), epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1).[46,47]

Of particular relevance for healing trajectory and for the ultimate scar phenotype are the infiltrating inflammatory cells and their biochemical signalers, as they exist in interaction with granulation tissue-resident cells as fibroblasts, myofibroblasts and endothelial cells.[44] Since cutaneous injury is linked to the release of ‘danger and pathogen signals’, innate immune receptors become activated and eventually trigger an inflammatory phase.[48]

The influx of polymorphonuclear neutrophils (PMNs) is ensured mainly by a complex cascade of vasoactive signalers and chemoattractants, so that these cells invade and are anchored within the wound matrix, initiating the wound’s acute phase that may last up to four days.[49] PMNs pattern recognition receptors are activated by local damage-associated molecular patterns (DAMP) released during cell injury and necrosis, and by bacterial pathogens’ associated molecular pattern (PAMP). Upon activation, these cells release pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interleukin 1 beta (IL-1 β) and IL-6, which amplify the inflammatory response and pave the way for macrophage infiltration and activation.[50] Activated PMNs ‘clean’ the wound bed of tissue debris via an armamentarium of degradative and antimicrobial proteases (cathepsins, defensins, lactoferrin and lysosomes) stored in cytoplasmic granules. This sanitizing process is largely dependent on the formation of neutrophil extracellular traps (NETs), web-like structures that capture and eliminate exogenous bacteria, fungi and viruses.[51] This process of NET release is termed NETosis and has broad implications for different forms of inflammation.[52] Recent studies show that circulating PMNs from diabetic subjects are biased toward excessive release of NETs,[53] and that uncontrolled NETosis impairs the healing process in diabetic mice and humans.[54] These cells also generate reactive oxygen species (ROS) that help eliminate invading pathogens.[55] Altogether, the role of PMNs in wound healing is undoubtedly important, but an extension of their local residence time and their functional profile may lead to wound chronicity (Figure 2).

Figure 2. Normal vs. diabetic healing



Disruption in phase progression, delaying normal progress.
NETosis: neutrophil extracellular traps release

A second generation of inflammatory infiltrating cells comes from local resident macrophages that are differentiated from infiltrating monocytes, which are activated via DAMP, PAMP and a cytokine surge.[56] Anti-macrophage depletion studies have revealed the physiological significance of these cells for the late stage of the inflammatory phase and for initiation of the proliferative one.[41,57,58] This late stage of the inflammatory phase is mostly characterized by the M1 subset of macrophages, which themselves are characterized by phagocytic and pro-inflammatory activities. Later, macrophages polarize to an M2 subpopulation with anti-inflammatory activity by expressing interleukin-receptor antagonists and a collection of fibroangiogenic growth factors that enhance fibroblast proliferation, extracellular matrix synthesis, angiogenesis,[59,60] ultimately reducing inflammation.[47] Macrophage subclass shift from M1 to M2 subsets is a meaningful event, given its role in turning off inflammation, clearing the wound bed of apoptotic PMNs (efferocytosis), ensuring proliferative phase progression, and preventing autoreactivity to released self-antigens.[15,57,61] Conclusively, inflammation is a time-restricted, finely controlled sequential event, whose expansion is paradigmatically associated with a torpid healing phenotype, or to wound chronification and senescence of granulation tissue productive cells.[16,17,62]

Granulation tissue is subsequently organized and populated by a broad spectrum of extracellular matrices, secreting cells that are in active and dynamic engagement with the substrate, and progressively modulating the structure and composition of the wound's extracellular matrix.[63] Although granulation tissue is a temporary organ, it is important as a 'welding material' filling wound gaps, preventing environmental threats, and providing support for cell adhesion, migration, growth and differentiation during wound repair.[63,64]

The proliferative phase also embraces three important processes: angiogenesis, wound contraction and re-epithelialization.

Angiogenesis is an exciting biological process actively regulated by pro-angiogenic growth factors, chemokines, integrin receptors, bone marrow-derived progenitor cells, and transcriptional and post-transcriptional epigenetic regulators; it restores blood inflow and outflow, and therefore oxygen delivery and CO₂ extraction. Its role in a normal healing trajectory is essential.[65] In response to pro-angiogenic signals like vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), PDGF-B, TGF-β, and angiopoietins, endothelial cells initiate angiogenesis by sprouting, proliferation and migration.[66] At the same time, locally secreted antiangiogenic factors are able to counterbalance and limit excessive angiogenesis.[67] The lack of an appropriate angiogenic response is a representative hallmark of DFUs, and identification of molecular forces underlying diabetic microangiopathy has been extensively examined.[68,69]

Wound contraction and epithelial resurfacing are two integrated mechanisms that ensure complete wound closure in most mammalian species. Contraction, a physiological and necessary event in wound closure, appears to be mediated by myofibroblasts, specialized cells responsible for force generation, that are differentiated from migrating fibroblasts during granulation tissue formation.[70] Although current theories posit that alpha smooth muscle actin-expressing myofibroblasts contribute to wound contraction,[71] others attest that contraction progresses through fibroblast-derived traction forces via thick collagen fiber secretion.[72] Irrespective of the contraction-responsible cell and the molecular drivers behind them, limited contraction is associated with torpid healing in diabetic wounds.[12]

Instrumental for complete and successful wound closure is re-epithelialization. This event demands integration of leading-edge keratinocyte proliferation, migration and differentiation in order to re-establish epidermal integrity.[73] This is perhaps one of the most complex and unexplored processes in wound healing.[74-76] Simplistically described, keratinocytes at the wound edge and epithelial cells from hair follicles in the vicinity migrate and proliferate. Signals that promote keratinocyte proliferation include heparin-binding EGF-like growth factor (HB-EGF), EGF, transforming growth factor alpha (TGF-α), and FGF secreted from platelets, macrophages and dermal fibroblasts.[77] For migration to progress, there is a reduction of desmosomes and hemidesmosome connection, cytoskeleton reorganization, morphological reprogramming and changes in the pattern of keratin expression.[78] In general, there is a loss of physical tension at points of cell attachment to the basal lamina.

Re-epithelialization progresses when the basement membrane is reconstituted by upper dermal fibroblasts and keratinocytes in a cooperative effort.[79] In this context, the interaction between integrin receptors and the neomatrix determines the speed of

migrating keratinocytes, a process that in turn is regulated by growth factor gradients.[80] Also important are plasmin and other plasmin-related degradative enzymes that degrade fibrin and other matrix glycoproteins, facilitating keratinocyte migration. Other collagenases and gelatinases are expressed by migrating keratinocytes.[77] Once keratinocytes attach to the basement membrane, they initiate a process of upward migration and differentiation to create a mature stratified, squamous epithelium that covers the wound.[81] Failed or delayed re-epithelialization is an obvious sign of wound stagnancy.[82] Compelling evidence documents the deleterious role of hyperglycemia and other downstream biochemical signalers on fibroblast and keratinocyte proliferation and migration.[83–85]

The ultimate phase—remodeling—begins approximately 14–21 days post-injury and may continue for years.[86–88] Its main function is the formation of normal epithelia and maturation of scar tissue;[89] excessive matrix is eliminated by a set of metalloproteinases, while new type-1 collagen fibers are produced and horizontally aligned in a more organized and esthetic manner. Inflammatory infiltration has ceased and different cell populations gradually enter into apoptosis.[90] The initial granulation tissue progressively collapses and is replaced by a new wound chemical milieu, so that the scar ECM architecture increasingly approaches original tissue morphology. The complexity involved in wound tissue remodeling has led us to hypothesize the existence of structural, positional and organizational memory in late wound cells, enforced by topographic ‘home address signals’. At this point, wound tensile strength is restored, and antiangiogenic mediators are locally released to turn off angiogenic sprouting and ensure excessive vessel regression. In parallel, other anatomical structures including the epidermis, nerves and myofibers are synchronously remodeled forming a functional unit.[78,86,91]

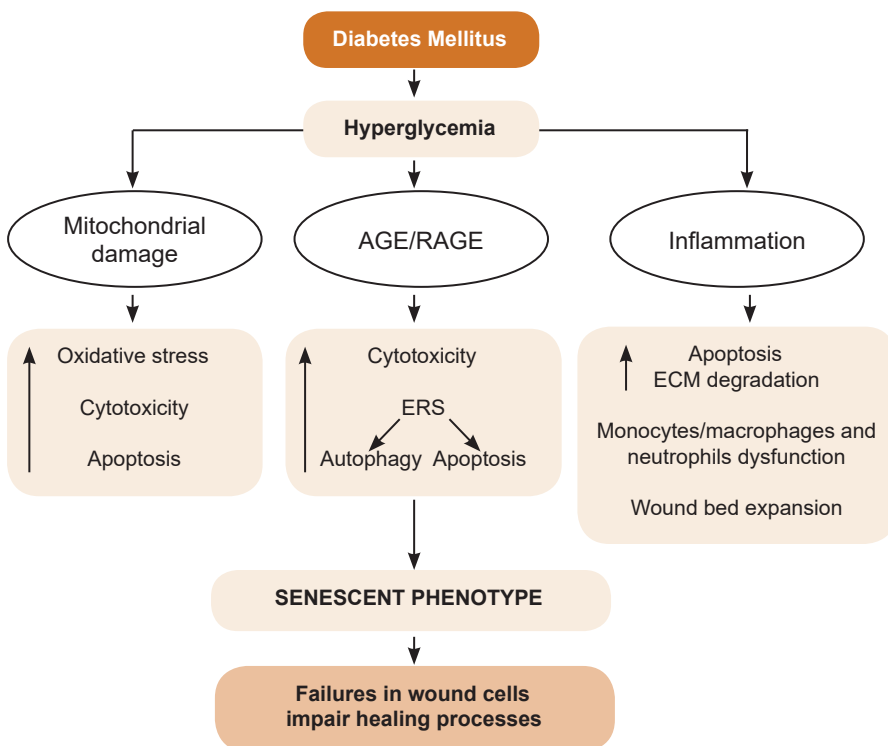
The diabetic foot ulcer: overview of the molecular bases of the torpid healing process Although it is generally accepted that time-to-heal determines a wound’s clinical classification as acute or chronic, the conceptual definition of wound chronicity has remained controversial.[92] Nevertheless, it is generally accepted that a wound is considered chronic when it fails to proceed in “an orderly and timely reparative process that results in sustained restoration of anatomic and functional integrity”. [92] DFU is archetypical of chronic wounds.[93–95] It is generally accepted that a primary hallmark of diabetic wounds is their persistent arrest in an unproductive inflammatory phase, associated with impaired formation and consolidation of mature granulation tissue.[96,97]

The arrest in this inflammatory phase is not associated with successful control of local infection, and thus it has been proposed that diabetic individuals are more vulnerable to wound infection[98,99] due to the existence of a primary deficit in innate immune response mechanisms.[100,101]

Hyperglycemia, again, seems to act as the proximal trigger for an exaggerated inflammatory reaction (Figure 3). Hyperglycemia and its distal operators—advanced glycation end products (AGE), TNF-α and other pro-inflammatory signalers—exert profound cytotoxic effects in fundamental ‘building-block’ cells of describes tissue.[11,102] Compelling evidence describes an inflammation-prone, pro-oxidative and pro-degradative environment in the core of diabetic wounds.[103–106]

Uncontrolled pro-inflammatory cytokine secretion imposes a pro-catabolic balance in the wound bed that both increases peripheral insulin resistance and reduces injured tissue’s anabolic response.[107,108] TNF-α downregulates fibroblast collagen synthesis in diabetic skin and upregulates the synthesis of metalloproteinases by amplifying the wound’s proteolytic and pro-degradative profile.[109,110] Although some studies have ruled out hyper- or hypoglycemia’s role in significantly disrupting PMNs cells’ ability to enter into apoptosis,[111] others have pointed to poorly controlled glycemia levels as a major factor in the prolonged residence of apoptosis-resistant PMNs with active secretory functions,[111] which ultimately translates into an elevated proteolytic/degradative balance.[112,113] This pro-degradative environment reduces local availability of growth factors and their receptors, hindering the ability of fibroblasts and endothelial cells to participate fully in the healing process. [88,114] PMN are also considered a source of ROS and nitric oxide species within the wound bed, with remarkable cytotoxic and pro-degradative potential.[115,116] The increased rate of ROS is an indirect consequence of poorly-controlled glucose levels given that existing evidence provides a connection between

Figure 3. Simplified hyperglycemia-associated healing impairment



High glucose burden and fluctuating glucose spikes are toxic under acute and chronic conditions to a large constellation of cell populations.
 AGE: advanced glycation end products ECM: extracellular matrix ERS: endoplasmic reticulum stress
 PMNs: polymorphonuclear neutrophils RAGE: AGE receptor

the high-glucose-induced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway and exaggerated NETosis.[117] Hyperglycemia also induces myeloid progenitor proliferation and expansion, as well as increased neutrophil production of S100A8/S100A9, which ultimately binds to AGE receptors (RAGE). This interaction translates to enhanced ROS production and myelopoiesis[118] that reinforces the triad of hyperglycemia, neutrophil infiltration and local ROS production.

Aside from PMN, M1 subclass macrophages predominate in diabetic wounds.[119] Although the molecular drivers behind this process are not yet fully understood, it has been demonstrated that human monocytes and macrophages undergo M1-like inflammatory polarization when exposed to high levels of glucose in both culture conditions and in hyperglycemic subjects.[120] This hyperpolarization to the pro-inflammatory arm represents a flaw in the transition to the M2 subclass and, therefore, to an anti-inflammatory and pro-healing profile. Additionally, diabetic wound macrophages exhibit defective efferocytosis, a mechanism for clearing apoptotic bodies in the wound bed.[121] This failure increases the local surge of proinflammatory cytokines, which perpetuates inflammation and increases the risk of it becoming chronic.[121] Another study has documented that hyperglycemia itself, without additional metabolic factors, induces a mixed profile of M1/M2 cytokines that nurture diabetes-associated inflammation and atherosclerosis.[122] The diabetic systemic low-grade inflammatory phenotype is able to introduce monocyte chromatin modification that, in turn, intensifies the persistent pro-inflammatory state.[123]

The proliferative phase in diabetic foot ulcer healing is frequently slow, torpid and asynchronous.[124] This may entail irregular and abnormal fibroblast recruitment, scarce or abnormal extracellular matrix protein secretion, limited cell-anchoring scaffold synthesis, poor or abnormal angiogenesis—including pathologic vascular remodeling, slow contraction of wound contours, torpid re-epithelialization and the inability to remain in remission after epithelial resurfacing.[11,125,126] From the molecular angle, compelling evidence has identified high glucose burdens and accompanying fluctuating glycemia spikes[127] as the proximal trigger of many cellular impairments that generically transform into fibroblasts, endothelial cells and keratinocytes in mitogenic and motogenic arrest; premature apoptosis and the onset of a senescent phenotype.[128–130] Multiple *de novo* circuitries, metabolic shunts, inflammatory-prone reactants and abnormal pathways in diabetics impact the wound-healing response and perpetuate the ulcer.[131,132]

Glycoxidation derivatives are intrinsically cytotoxic to productive cells in granulation tissue, and further amplify pro-inflammatory and pro-oxidative circuitry by binding to RAGE.[133,134] Glycoxidative products accumulate in non-labile dermal collagen[132,134] leading to cutaneous cell toxicity and premature senescence, impairing fibroblast and endothelial cell physiology,[99] and consequently delaying granulation tissue formation and maturation.[11,133] Conclusively, within the wound, the triad of TNF- α , ROS and AGE can initiate apoptosis of fibroblasts and vascular cells, thereby prolonging inflammation, reducing growth factor availability and opening the gate for the onset of the so-called 'wound senescent cell society'. [130,135–137] It is not surprising therefore, that repair-committed cells in diabetics move through proliferative arrest, senescence, and apoptosis (Figure 3).[136]

Re-epithelialization failure in diabetics and the tendency toward local recidivism are significant challenges for clinicians, wound care providers, and basic scientists. It has been suggested that an incomplete program of keratinocyte activation and differentiation[138] is fundamental for the presence of mitotically active—but not migrating—epithelial cells along the wound's leading edge.[73,76] High glucose has shown to exhibit a toxic effect on keratinocytes, reducing proliferation, replicative life span,[139] and migratory responses.[140] The fact is that, as long as the wound is not resurfaced, the threat of infection and amputation remains.

Diabetes and infection susceptibility The relationship between DM type 2 (DMT2) and immunity is an expanding research field in which new puzzle pieces are continuously discovered, often increasing in complexity and stimulating controversy within the field. This research incentive is fueled by the understanding that diabetes increases the risk of certain infections[141] and infection-related mortality.[142]

DMT2 is currently considered an immunometabolic disease, given the role of T-lymphocyte activation in inflammation and in the onset of insulin resistance.[143] The robust pathogenic loops linking insulin secretion, peripheral insulin resistance, immunoinflammation and DMT2 are beyond the scope of this analysis, and have already been thoroughly reviewed.[144–146] The links are well defined: inflammation leads to peripheral insulin resistance and, in turn, insulin resistance leads to inflammation.[147] However, it is important to note that inflammation does not necessarily represent immunocompetence. On the contrary, some experimental data suggest that diabetes-associated hyperinflammation amplifies damage from bacterial infections and leads to increased susceptibility to Gram-negative bacteria.[148] Since the actual cause of death of the mice in one study was hyperinflammation, the authors suggest that this rather counterintuitive finding may respond to diabetes-associated RAGE overexpression, which preconditions a chronic inflammatory scenario that ultimately may be lethal when presented with the additional challenge of a bacterial infection.[138] Cumulative evidence documents a significant correlation between infection and rate of glycemic control.[21,149,150] It follows that heightened susceptibility to infections would be associated with insufficient glycemic control.[151,152] This observation is particularly relevant in the scope of our review to cellulitis, DFU, the devastating conditions of necrotizing fasciitis, and Fournier's gangrene.[22]

Hyperglycemia and insulin deficiency are considered the two major etiopathogenic pillars of diabetes-associated immunodeficiency (Figure 4) and susceptibility to infections.[153–155] Hyperglycemia as a primary trigger of pro-inflammatory cytokine spillover[145] results in local and systemic inflammation, and peripheral insulin resistance.[156,157] Increased levels of various inflammatory markers and mediators—including white blood cell count, C-reactive protein, pro-inflammatory cytokines and plasminogen activator inhibitor-1—are elevated in insulin resistant subjects and DMT2-affected patients.[158,159] Although it may appear contradictory, evidence suggests that peripheral blood mononuclear cells (PBMC) from DMT1 and DMT2 patients secrete lower constitutive-[160] and lipopolysaccharide (LPS)-stimulated[161] levels of TNF- α , IL-1 (α and β) and IL-6, as compared with matched controls. The same cytokine secretion impairment was confirmed for in vitro models where PBMCs from healthy donors

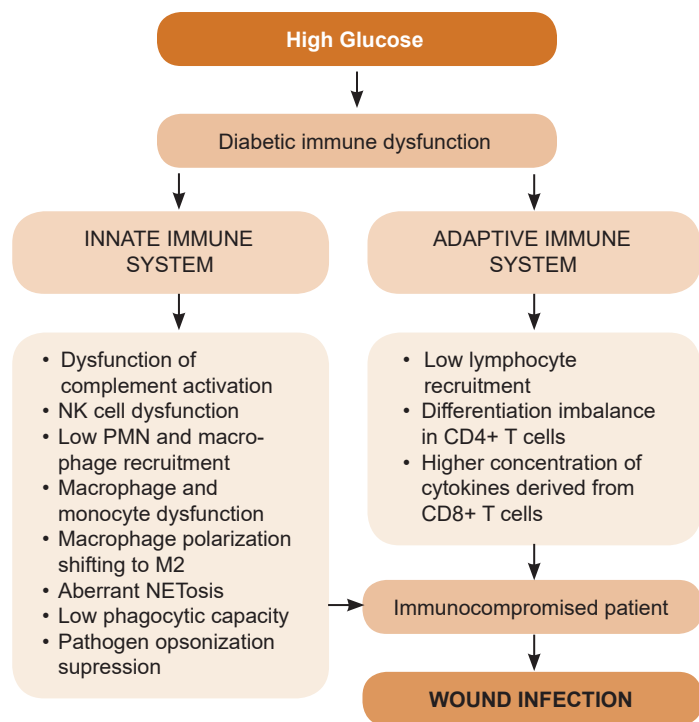
were exposed to high glucose levels[162] or dextrose octreotide.[163] This study demonstrates that glucose exposure dampened IL-2, IL-6 and IL-10 levels in a concentration-dependent manner while conversely inducing the expression of TGF- β 1 which may explain immune failure.[162]

Increased glycation leads to a reduction of IL-10 secretion by myeloid cells.[32] A recent study demonstrated that PBMC steady-state expression of IL-1 β appeared significantly increased while IL-6 expression reduced 3.45-fold in a cohort of DMT2 patients, compared with healthy control subjects.[164] This is a counterintuitive observation considering the canonic links between IL-6, glucose metabolism, DMT2 and their complications.[165–168]

Aside from these findings, a wealth of classic studies associated elevated endovascular levels of pro-inflammatory cytokines with insulin resistance and DMT2.[169,170] Subclinical blood elevation of some of these markers anticipates the onset of DMT2,[171,172] and the progression of multi-organ complications.[173,174] More recent studies[175] have identified and expanded epigenetic explanations as to why hyperglycemia induces long-lasting inflammatory upheaval, even days after glucose normalization. The fact is that hyperglycemia-associated chronic inflammation is epigenetically sculpted and is one of the biochemical insignias of metabolic memory.[176] Therefore, unraveling the roots of this plethora of conceptual controversies requires additional studies.[177–179]

Incorporation of AGEs to non-diabetic-derived cells has conclusively shown elevations in cytokine secretion,[100]

Figure 4. Diabetes impairs immune response and predisposes patients to wound infection



Hyperglycemia disrupts both the innate and the adaptive immune systems, making diabetic subjects susceptible to infections.

NETosis: neutrophil extracellular traps release

NK cells: natural killer cells

which led to the hypothesis that AGE is somehow involved in diabetics' increased basal cytokine secretion.[100] Glycation also reduces expression of class-I major histocompatibility complex on the surface of myeloid cells, which accounts for impaired cellular immunity.[32] The presence of high glucose concentrations leads to elevated constitutive (steady state) cytokine production in resting cells, which becomes insufficient upon PBMC stimulation. Cell stimulation provoked by cytokine insufficiency is thought to be a critical factor in impaired immunity and vulnerability to invading pathogens among diabetics.[32,162,163,180] However, an alternative line of thought has suggested that stimulating the immune system will imperil—rather than protect—diabetics from acute Gram-negative bacterial infections, and that dampening hyperinflammation (with dexamethasone, for example) may restore the innate immune response to such infections.[148]

Other studies have addressed leukocyte recruitment and pathogen recognition abilities. Animal models demonstrate that diabetes impairs CD45+ leukocyte and CD8+ T cell recruitment, and reduces adhesion molecule expression and cytokine production upon microbial invasion of different organs.[181,182] More conflicting evidence exists, however, regarding pathogen recognition via toll-like receptors (TLR). One line indicates that TLR expression is reduced in diabetic mice.[182,183] In contrast, studies in human samples attest that TLR expression is low in diabetic subjects with complications and deficient glycemic control, whereas it is high in patients with well-controlled glycemia without complications.[184] Overall it has been concluded that TLR pathway impairments lead to diminished recognition of bacteria and may be one of the mechanisms implicated in diabetic susceptibility to wound infection.[183]

More coincidental revelations stem from the characterization of neutrophil dysfunction documented in diabetic individuals or in healthy donors exposed to high glucose burdens. The so-called 'oxidative burst' plays a critical role in neutrophils' antimicrobial defense and is reduced upon high glucose concentration due to poor ROS and superoxide anion productions.[185,186] Other studies have documented that sustained hyperglycemia leads to neutrophils' functional decline and that the mechanisms behind this decline include increased adhesive capacity, as well as diminished chemotaxis, phagocytic activity and bactericidal capacity.[102,187,188] This is a conflicting observation given the solid evidence supporting the concept that hyperglycemia is a major trigger of inflammation and hyper-oxidation in diabetes.[189–191]

Other studies show lower neutrophil degranulation,[192] decreased phagocytic capabilities,[193] immunoglobulin-mediated opsonization inhibition[194] and limited NET response ability.[195] These conflicts could be due to the type and origin of the experimental designs of, and samples used in, the studies; however, the overall interpretation of these data has led to the conclusion that hyperglycemia dampens PMN chemotaxis and phagocytic activities.[21] Diabetes impairs the physiology of macrophages and other innate immune cells. As stated above, diabetic patients are highly susceptible to bacterial infections, and often have impaired wound healing. However, despite years of research, the

molecular mechanism underlying macrophage dysfunction in diabetes is not fully understood .[196]

Under high glucose conditions, in vitro and in vivo models have shown reduced complement receptors, adhesion capacity, phagocytosis and antibacterial activity.[197] Diabetic hyperlipidemia and hyperglycemia introduce epigenetic modifications to macrophages that promote the onset of an inflammatory phenotype. [198] Thus, high glucose levels induce inflammatory polarization of human macrophages in vitro whereas AGEs significantly prime and promote M1 macrophage markers expression and IL-6 and TNF- α secretion.[199] Accordingly, M1 macrophages have strong microbicidal and antigen-presenting capacities, produce pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β), and ROS; whereas M2 macrophages are considered pro-resolution response cells, producing anti-inflammatory mediators (IL-10 and TGF- β) and consequently resolving inflammation.[200] In light of these observations, it is controversial to posit M2 macrophage polarization in diabetic mice (db/db) as an explanation for their susceptibility to bacterial infection.[196] As a matter of fact, M2 cells are scarce within the wound environment where they are obviously necessary.[201] Irrespective of the controversial issue of in-wound recruited macrophage polarization, a recent study confirms that in diabetic mice, macrophage phagocytosis and bactericidal activities are reduced upon long-term exposure to high glucose burdens.[196] For these authors, long-term high-glucose treatment reduced macrophage glycolytic capacity and glycolytic reserve, in turn, impairing phagocytic capability.

NK (natural killer) cells derived from diabetic individuals also demonstrate defects in activating NKG2D and NKp46 receptors related to NK degranulation failure.[196] Hyperglycemia is also associated with a reduction in C4-fragment opsonization, which inhibits classical or lectin pathways of complement activation,[202] in impaired neutrophils' bacterial killing capacity.[100,203] By using peripheral blood lymphocytes from diabetic animal models and human samples, studies have concluded that uncontrolled diabetes increases chromatin condensation, DNA fragmentation and lymphocyte death.[204]

Unlike the effect of hyperglycemia on immune cell activity in DMT2, the impact of insulin deficiency in DMT2 immunoresponsive cells against pathogens has not been widely studied.[170] Given that these cell functions are energy-dependent processes, proper insulin-regulated glucose metabolism is necessary. Insulin-driven metabolic processes are not merely associated with immune-cell ATP generation and utilization. Glucose and lipid metabolism influences cellular phenotype, potential cellular reprogramming during patterns of recognition, and ultimately activation status.[205] In activated T-lymphocytes, insulin stimulates glucose uptake, oxidation, pyruvate flux and pyruvate dehydrogenase activity, amino acid transport, lipid metabolism and protein synthesis.[206] Recent findings implicate insulin in shaping the immune response by modulating cell differentiation and polarization.[207,208] Thus, in addition to its role in substrate metabolism, insulin is also an anti-inflammatory and immunomodulatory hormone[209,210] via immune cells' metabolic regulation.[211]

Recent studies substantiate the importance of insulin in normal innate immune response. An insulin deficit is associated with alveolar macrophages' phagocytic impairment as well as poor cytokine secretion in alloxan-treated rats, reverted after insulin

intervention.[212] Insulin treatment of diabetic mice bone marrow-derived macrophages restored production of critical pro-inflammatory cytokines upon LPS exposure.[213]

Conclusively, the apparently 'trivial' blood glucose derangements in diabetes reduce bactericidal and wound healing capacities in innate immune operators, a phenomenon that, according to latest evidence, is related to transcriptional aberrations in gene coding for macrophage differentiation and lymphocyte migration and proliferation at the hematopoietic stem/progenitor cell level.[214] Thus, therapeutic manipulation of immune-metabolomic loops is a promising therapeutic road.

Infection of diabetic foot ulcers: biofilm and its interaction with the wound matrix Typically, once an ulcer develops, it is colonized with microorganisms that may lead to a state of clinical infection.[113] About 15%–25% of DM patients develop foot ulcers during their lifetime and half of these become infected, a recurrent complication in diabetics.[215,216] Infection can spread to soft tissues and bone making it the main causal factor of lower extremity amputation in most countries.[217] Thus, the management of DFI represents a high cost for the health system, a decrease in the quality of life of diabetic population, and a great research incentive.[217]

DFI is defined as the presence of an inflammatory response and tissue damage that can drive the clinical spectrum from superficial cellulitis (mild infection) to chronic osteomyelitis (severe infection), with host–microorganism interaction being crucial in determining progression.[218,219] This interaction is defined by Casadevall and Pirofski as the “damage response framework model”, [220] and proposes that infection outcomes are dependent on mutual contributions of both the microbe and the host.[220] In comparative terms, infection occurs when invading organisms overwhelm the host's defenses.[221] In contrast, colonization is defined as the presence of proliferating bacteria without an overt host immunological reaction.[26] The reported critical limit is 10^5 colony-forming units per gram of tissue,[222] indicating the presence of a 'critical' degree of colonization marking the point at which host defenses are no longer able to contain the infection.[223] Nevertheless, preexisting diabetic neuropathy, peripheral vascular disease, impaired leukocyte function[224] and a deteriorated innate immune system make DFI clinical diagnosis difficult while simultaneously worsening its prognosis. [225,226] Under these circumstances, onset of classic signs of infection may not occur,[227] even when there is a high bacterial load.[226] Thus, the invading pathogen may progress and infect with no clinical translation.

Staphylococcus aureus is a major pathogen of human skin. This is also the most common pathogen identified in patients with acute superficial DFU.[23,228,229] This pathogen, in its interaction with the host's diabetic wound environment, is able to amplify certain glucose-regulated genes that ultimately increase its virulence, indicating that hyperglycemic conditions facilitate pathogen adaptation and survival, ultimately worsening patient prognosis.[230]

Chronic ulcers usually exhibit polymicrobial infections including a mixture of aerobic/anaerobic and Gram positive/negative bacteria.[227,231] Some of the heterogeneous groups of bacterial species identified in DFU patients have been compiled

in Table 1.[26,232–234] Bacterial predominance differs between studies. Nevertheless, recent literature point to Gram-negative bacteria as the predominant group.[23,229,235,236] The reported prevalence included *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*. [237,238] Usually, these results are influenced by several factors including infection severity, demographic characteristics, glycemic control, and ongoing or previous antibiotic treatments, as well as bacterial identification method.[26]

Table 1: Bacterial species identified in diabetic foot ulcers

Aerobic and anaerobic facultative bacteria	Anaerobic bacteria
<i>Staphylococcus epidermidis</i>	<i>Clostridium species</i>
<i>Staphylococcus saprophyticus</i>	<i>Peptostreptococcus species</i>
<i>Pseudomonas aeruginosa</i>	<i>Dialister pneumosintes</i>
<i>Klebsiella pneumoniae</i>	<i>Bacteriodes fragilis</i>
<i>Escherichia coli</i>	<i>Anaerococcus prevotii</i>
<i>Streptococcus mutans</i>	<i>Anaerococcus tetradius</i>
<i>Streptococcus pyogenes</i>	<i>Eggerthella lenta</i>
<i>Bacillus subtilis</i>	<i>Fusobacterium mortiferum</i>
<i>Proteus species</i>	<i>Veillonella dispar</i>

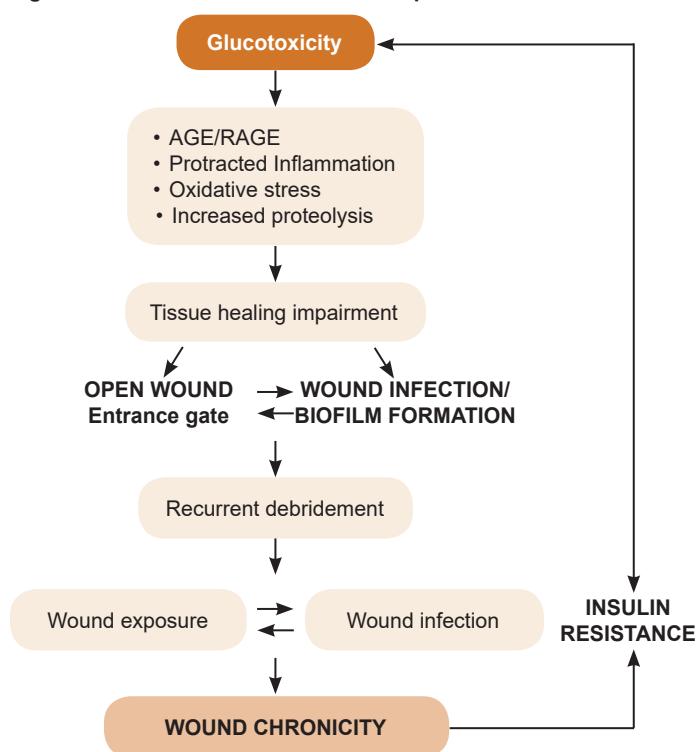
*Adapted from [26] and [234]

A traditional debate in this field is the relationship between pathogenic potential and microbial bioburden, and how it impacts the host. Here, it is important to highlight the diversity of the microorganism population and its potential interactions, as these may turn into cooperative pathogenic loops that enhance antimicrobial resistance and imprint a particular signature on each individual ulcer.[239] The symbiotic microbial interaction within the ulcer’s ecosystem confers a virulence profile that is far more important than the microorganism concentration in and of itself.[222,240] Long-standing ulcers are more predisposed to infection which, in addition to impairing the healing response, may reduce peripheral insulin sensitivity.[21,241,242] Thus, the longer the wound is maintained, the greater the risk for infection. Another contribution of infected ulcers is their pathogenic effect at the organism level. These ulcers act as pro-inflammatory organs superimposed onto a host, pouring pro-inflammatory reactants, oxygen free radicals and bacterial toxins into central circulation, amplifying tissue injury and general homeostasis (Figure 5).[243]

Finally, it is noteworthy that DFI is frequently associated with existing biofilm in the context of ulcers. Biofilm is a niche for symbiotic microorganism interactions that essentially act as a protector shield for bacterial populations.[244–246] How biofilm-making microorganisms interact with immunocompromised diabetics and their subsequent pathophysiological consequences are relevant research topics.

The term biofilm was coined by the scientific community at the end of the 20th century, which indicates that this is an emerging and expanding research area.[247–250] DFU pathogens can exist as planktonic form (free-living) or as a biofilm (sessile-living).[246] Both phenotypic states may play important roles in impairing healing and causing infection of both acute and chronic wounds.[251] Biofilm acts as a collective entity endowed with superior antimicrobial resistance when compared with its individual constituents. Several in vitro experiments indicate that antibiotic resistance in biofilm bacteria is up to 1000 times higher than in planktonic bacteria.[252]

Figure 5. Wound–infection feedback loop



AGE: Advanced glycation end products RAGE: AGE receptor

The ability of a microorganism to build up biofilms is an important virulence factor and an advantageous organizational step. As stated above, biofilm offers a protective environment or physical barrier to biological and antimicrobial substances, facilitating microorganism attachment to surfaces or to each other, ultimately enabling survival and antibiotic resistance.[236,245] ‘Inoffensive’ non-pathogenic bacteria, incapable of promoting chronic wound infection, may symbiotically interlink with pathogenic biofilm and act synergistically to cause a chronic infection.[24] This structured community of microorganisms can be classified as mono- or polymicrobial, encased in extracellular polymeric substances (EPS) or exo-polymeric substances.[253]

The community is a mixture not only of bacterial cells, but also of fungi, viruses, proteins, extracellular DNA and other biogenic factors that increase virulence and reduce treatment success.[246] This and other virulence factors are possible through cell-to-cell communication via quorum sensing (QS).[246,254] QS is a form of cellular communication mediated by small molecules that depend on cell density. The species of bacteria that reaches critical-mass concentration produces large amounts of small signaling molecules that modify gene expression. Indeed, bacterial exchange coordination activities are based on this mechanism, according to population size.[246] QS, together with the exchange of genetic material by bacteria in the biofilm, give rise to different microorganism phenotypes that ultimately affect ulcers as can be seen in anti-microbial treatment results.[23] The fact that bacteria are not motile in the biofilm context and have lower metabolic and proliferative activities than their planktonic counterparts,[255] makes appropriate antibiotic selection difficult. Many antibiotics used for DFI treatment are only effective against actively dividing cells.[256]

Over the last few years, the concept of biofilm in dynamic reciprocity between wound-bed cells and the host has attracted increasing interest. Correspondingly, it has been proposed that biofilms are responsible for over 90% of all chronic wounds. An electron microscopy study assessing wound tissue biopsies suggested that about 60% of chronic wounds have a biofilm compared to 6% of acute wounds.[249] It is likely that at least half of all chronic wounds develop a biofilm.[248,249] This result indicates the contribution of biofilm to impaired wound healing even when molecular mechanisms underlying biofilm-induced chronification remain poorly understood.[257,258]

The various mechanisms by which biofilm obstructs the healing response include failures in granulative tissue formation and the re-epithelialization trajectory.[259] Accordingly, it is likely that these events are consequences of anti-proliferative signals derived from pathogens and a persistent inflammatory environment[257] that aborts fibroblasts and keratinocyte mitogenic, motogenic and secretory functions.[260] In line with this notion, Trøstrup demonstrated that *P. aeruginosa* induces a state of cellular quiescence reminiscent of premature senescence.[62] *P. aeruginosa* also secretes a plethora of proteases resulting in collagen, fibrinogen and elastin degradation, inhibition of PMNs and complement systems, and basement membrane degradation.[261,262] Similarly, proteases secreted by *S. aureus* also degrade collagen and elastin. The ability to degrade surface-associated adhesins enables bacterial phenotype transition from adhesive to invasive.[263] Inhibition of neutrophil phagocytosis and chemotactic activity is also associated with bacterial wound infection.[264] In this steady inflammatory milieu, PMN-derived elastase and other degradative enzymes increase wound tissue damage, expand wound size and perpetuate chronicity.[62] In-depth studies examining *P. aeruginosa* and host interaction showed that TLR activation is inhibited by pathogen-derived elastase, which allows evasion of host immunosurveillance.[265] *P. aeruginosa*-derived rhamnolipids inhibit human beta-defensin secretion by challenged keratinocytes, which contributes to pathogen survival and colonization in compromised epithelia.[266] Furthermore, it has been proposed that biofilm and lipopolysaccharide EPS of Gram-negative bacteria inhibit complement activation, further contributing to evasion of the host's innate defense system.[267,268] Microorganism-derived PAMP, together with platelet-derived factors stimulate the influx of PMNs and other immune cells, spreading the wound's pro-inflammatory reactants, increasing the level of ECM-degradative proteases, and consequently curtailing the proliferative phase.[112,269,270] Additionally, infiltrated inflammatory cells in response to bacterial invasion via proinflammatory cytokines and AGE/RAGE axis activation produce large amounts of ROS that act as local causal factors for premature cell senescence.[271,272] In other words, the pathogen manages to prevent otherwise normal PAMP-induced innate immunity activation; and graphically speaking, the DFU turns into a battlefield in which the pre-debilitated diabetic host's immune system is overwhelmed by biofilm-entrenched microorganisms, thus perpetuating wound arrest.[62,273]

Biofilm identification is complex and dependent on more than simple wound cultures obtained and evaluated using traditional microbiological techniques. More sophisticated and expensive techniques such as light and scanning electron microscopy are required to evaluate biofilm in a wound. Therefore, biofilm presence is often overlooked.[274,275] New molecular techniques

including DNA micro-arrays, multiplex real-time polymerase chain reaction and functional metagenomics offer a unique opportunity to characterize biofilm microbiome.[218] These technologies facilitate analysis of a microorganism's resistance potential and virulence factors.[26]

Conclusively, underlying diabetic complications predispose increased risk of developing DFI and other peripheral tissue infections as compared to the risk in healthy populations. Biofilm-forming microorganisms counteract the host's defenses, prolong DFU inflammation, deteriorate host anabolism and ultimately increase the risk of amputation (Figure 5).[24,258,276]

CONCLUDING REMARKS

Wound healing is a complex biological process consisting of precisely-predetermined overlapping phases integrated in a sequential cascade aimed at morpho-functional restoration in a physiological time window. Successful reparative response requires concerted and cooperative integration of both systemic and local signaling networks and driving forces. DM is an archetypal disease in which a variety of both local and systemic factors combine to disturb most healing phases. Thus, DFUs serve as a model for chronic wounds, an often-devastating diabetic complication, and the first cause of lower-limb amputations worldwide.


Underlying the ulcer's onset and expansion is a complex interplay of pathogenic vicious circles that turn DFUs into a pro-inflammatory, pro-oxidant, pro-apoptogenic and pro-senescence-inducing organ, superimposed onto a host with an already-debilitated immune response. The failure of diabetic patients' peripheral immunosurveillance, as in the subsequent elicitation of effector mechanisms, is the foremost contributing factor to DFU infections.

Diabetic dysimmunity is likely a major determining factor on patient outcomes. It seems there is still a long way ahead before we achieve a uniform, comprehensive understanding of the actual immune profile of diabetic individuals. Further studies are needed to disentangle critical contradictions and contemporary conceptual paradoxes. Some of the current critical controversies are highlighted in this paper. Of note, however, is the divergent experimental data on whether hyperglycemia reduces pro-inflammatory cytokines and whether this cytokine dampening accounts for immune failure, as compared with other major ongoing questions in DMT2.

We still do not know how to manipulate hyperinflammation or how to reinstate leukocyte physiology once it is disrupted. At the moment, infection remains a dismal complication of these wounds, protracting pre-existing inflammation, dismantling local immune response, amplifying fibroblast and keratinocyte arrest and further disrupting the host's internal homeostasis. Given that DFUs are recurrently seeded by biofilm, eliminating infection is a challenging task. This review confirms that, despite the efforts to understand DFU pathophysiology, infection pathology and its interaction with the host, DFU prevalence is rising while any reduction in amputation rates remains modest. Therapeutically promising targets include: 1) Identification and pharmacological manipulation of epigenetic drivers in wound-prolonged inflammation and chronification, even in the ideal scenario of a non-infected ulcer; 2) Ablation of the wound cell-senescent drivers, which could contribute to restoring an acute wound-like closure trajectory; and 3) Characterization of actors and pathways underlying hyperglycemia and insulinopenia-

induced diabetic immune failure, and subsequent pharmacological interventions to rebuild the immune system. These academic imperatives must go hand-in-hand with diabetic self-care educational programs, as well as systematic foot and neurological examination by qualified specialists.

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